

The Role of the First Quartet Micromeres in the Development of the Polyclad *Hoploplana inquilina*

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Abstract. The role of the first quartet micromeres at the eight-cell stage in the development of the polyclad flatworm *Hoploplana inquilina* was analyzed with regard to specific contributions made by these cells and their function in the determination of embryonic symmetry. The experimental series involved: (1) deletion of one micromere (1a or 1c versus 1b or 1d); (2) deletion of two adjacent micromeres; (3) deletion of three micromeres; (4) isolation of intact first quartets; and (5) isolation of macromere sets 1A–1D. As the number of micromeres removed was increased, the larvae became progressively more abnormal, involving reduction in number of eyes, deficiencies in lobe development, and disturbance of embryonic symmetry. After deletion of three micromeres, none of the larvae exhibited normal morphology. These experiments indicate that the determination of embryonic axes leading to a larva with bilateral symmetry may involve micromere-macromere interactions, as has been shown in molluscan embryos with equal cleavage. Isolated first quartets consistently formed spherical, bloated, transparent larvae with multiple eyes, suggesting that the macromeres play an inhibitory role in eye development. Isolated macromeres 1A–1D often failed to develop, and larval structures never differentiated. Thus, the relatively loose determination of the polyclad embryo involves both cytoplasmic localization and cell interactions.

Introduction

The establishment of cell fate has been investigated in a number of animal species having embryos that exhibit spiral cleavage. Much of our current understanding of the role that cytoplasmic determinants play in embry-

onic development derives from studies of higher Spiralia, which typically express early embryonic determination or mosaicism. Such investigations have revealed complexity and variety within these fundamentally mosaic systems. For example, cell interactions have been demonstrated in many spiralian embryos, and within the molluscs, two different mechanisms of achieving the embryonic axes of symmetry have evolved.

Some turbellarian platyhelminthes have spiral cleavage, and they are more primitive than the annelids and molluscs on which these investigations have been carried out. Therefore, studies of turbellarian development should provide insights, not only into the nature of cytoplasmic localization, but also into the origin and evolution of spiral cleavage and embryonic determination. The polyclads, in particular, have a typical quartet spiral cleavage that is strikingly similar to the pattern in annelids and molluscs, but there has been little experimental work done on the development of this group.

The normal development of a polyclad flatworm has been most thoroughly examined in *Hoploplana inquilina* (formerly *Planocera inquilina*) by Surface (1907). Cleavage follows the typical quartet spiral pattern in which the macromeres (A–D) divide to produce four quartets of micromeres. At the four-cell stage, the embryo consists of two equal-sized A and C blastomeres that are displaced toward the animal pole, and two equal-sized but larger B and D blastomeres that are in contact at the vegetal cross-furrow. [Surface (1907) reports that the D cell is larger than B, but I have not observed this to be the case.] The eight-cell stage (Fig. 1A) consists of the animal first quartet (1a–1d) and the vegetal macromeres (1A–1D). Later, when the cells of the fourth quartet (4a–4d) are produced, they are significantly larger than the macromeres (4A–4D) and contain most of the

yolk. This is characteristic of the polyclads, distinguishing them from other spiralian. Gastrulation proceeds primarily by epiboly of the micromeres over the macromeres. By the fourth day, the embryo begins to rotate, the lobes have started to form, and differentiated tissues are discernable. A fully developed Muller's larva (Fig. 1B) is produced by the fifth day.

The embryos of *Hoploplana* have the characteristic mosaic development of the Spiralia (Boyer, 1986, 1987). Blastomere deletion and isolation experiments on two- and four-cell embryos produced partial larvae with characteristic deficiencies associated with each type of experiment. "Half larvae," resulting from the separation or deletion of two-cell embryos, were abnormal in body shape, and in the development of lobes and eyes. Deletion of one cell at the four-cell stage produced less anomalous "three-quarter larvae" that were underdeveloped in one quadrant and often exhibited eye abnormalities. These results resemble those obtained from such experiments on higher spiralian—that is, development is fundamentally mosaic—but the studies did not include analysis of embryos beyond the four-cell stage, and the mechanism of determination of the embryonic axes of symmetry was not examined.

Experimental analysis of eight-cell stage (first quartet) embryos of some of the higher spiralian have focused on two different problems, the cytoplasmic contributions of the first quartet cells, and the role that these cells play in the determination of embryonic symmetry. Among the former studies are those of Wilson (1904) on *Patella*, Horstadius (1937) on *Cerebratulus*, Costello (1945) on *Nereis*, Clement (1967) on *Ilyanassa*, Morrill *et al.* (1973) on *Lymnaea*, and van Dam and Verdonk (1982) on *Bithynia*. In general, the results supported the concept of mosaic development, though there was evidence of some regulative capacity in *Bithynia* and *Lymnaea*.

Determination of the embryonic axes of symmetry has been extensively studied in gastropod molluscs, a group in which two different mechanisms are involved. In gastropods with unequal cleavage, axis determination has occurred by the four-cell stage. At this time the D blastomere, which is larger than the others, inherits cytoplasmic determinants specifying mesoderm and the dorsal quadrant. Thus, by the four-cell stage, the axes of bilateral symmetry have been established through an intracellular mechanism involving cytoplasmic localization (see Davidson, 1986, for review), and deletion of the first quartet at the eight-cell stage does not affect determination of the dorso-ventral axis (van Dam and Verdonk, 1982).

In gastropods with equal-sized blastomeres at the four-cell stage, the first quartet cells play a crucial role in establishing the embryonic axes of symmetry (van den Biggelaar, 1976, 1977; van den Biggelaar and Guerrier, 1979;

Arnolds *et al.*, 1983; Martindale *et al.*, 1985). These embryos remain radially symmetrical with equipotent quadrants until after formation of the third quartet. At this time, the first quartet micromeres contact the central macromere and induce it to become dorsal (van den Biggelaar, 1976, 1977). If these interactions are delayed (Martindale *et al.*, 1985) or inhibited (van den Biggelaar and Guerrier, 1979), no D quadrant forms, and the embryo remains radially symmetrical. Thus, in these molluscs, intercellular interactions determine the embryonic axes of symmetry.

That such closely related groups should have such different mechanisms for establishing the D quadrant is surprising, because the cleavage patterns and cell lineages of these animals are very similar, and both equal and unequal cleavage occur widely in the molluscs. As Martindale *et al.* (1985) state, "We need to know more about the experimental embryology of other spiralian before the significance of the differences in the mechanisms which have been uncovered so far can be put into perspective." Because the ancestral flatworms may have been the first animals to evolve bilateral symmetry, the mechanism of symmetry determination in this group is particularly significant.

The *Hoploplana* embryo, with two equal sized A and C blastomeres and two larger but also equal sized B and D cells at the four-cell stage, does not appear to fit either model. The consistent pattern of defects occurring with deletion experiments on two- and four-cell embryos indicates that morphogenetic determinants specifying particular larval structures are organized in the zygote, but *Hoploplana* does not appear to have a designated cell corresponding to the D blastomere of higher spiralian. Therefore, I have examined the eight-cell (first quartet) stage to determine not only the specific cytoplasmic contributions of the micromeres, but also whether their interactions with the macromeres are involved in establishing the axes of symmetry.

Materials and Methods

Specimens of *Hoploplana inquilina* were collected from the mantle cavity of *Busycyon* and maintained in seawater in finger bowls. Gametes were removed from the spermatid vesicles and uterus by piercing the organs with sharp needles. Fertilization, which produced naked zygotes that lack the egg-shell membrane, occurred when eggs and sperm were mixed in plastic petri dishes containing Millipore-filtered seawater.

The blastomere deletion experiments were done by puncturing the selected cell with hand-pulled glass needles, typically about one half hour after cleavage to the eight-cell stage. The experimental embryos were examined carefully to be certain that each punctured cell had

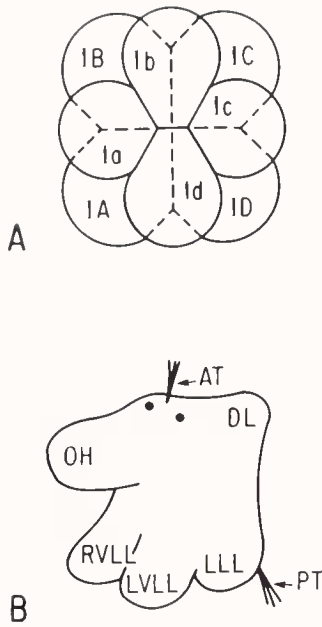


Figure 1. Normal developmental stages of *Hoploplana*. (A) Eight-cell stage viewed from animal pole. (B) Muller's larva. AT: apical tuft; DL: dorsal lobe; LLL: left lateral lobe; LVLL: left ventrolateral lobe; OH: oral hood; RVLL: right ventrolateral lobe; PT: posterior tuft.

cytolyzed completely. If deletion was incomplete, the embryo was discarded. The experimental series involved: (1) deletion of first quartet micromeres 1a or 1c *versus* 1b or 1d (it is not possible to differentiate between the A and C or the B and D quadrants); (2) deletion of two adjacent micromeres; (3) deletion of three micromeres; (4) isolation of the first quartet intact by killing the macromeres; and (5) isolation of the 1A–1D macromeres intact by deleting the first quartet.

Experimental embryos were raised in Millipore-filtered seawater to which 100 units/ml penicillin and 200 $\mu\text{g}/\text{ml}$ streptomycin were added. They were examined daily and analyzed on day six or seven for abnormalities in eye numbers, tufts, and morphology.

Results

The larvae became progressively more abnormal as the number of deleted micromeres increased. If larval morphology appeared normal, the larvae were categorized as Muller's larvae (Fig. 1B). If they exhibited any abnormalities similar to those resulting from deletion of one cell at the four-cell stage, such as absence or truncation of one or more lobes, they were termed "three-quarter larvae" (Fig. 2A). Larvae that were underdeveloped, asymmetric (lacking the normal axes of bilateral symmetry), and unrecognizable as Muller's larvae, were similar to those produced by deletion of one cell at the two-cell stage, and were called "half larvae" (Fig. 2B). A fourth

category, not normally a product of deletion experiments on two- and four-cell embryos, appeared with deletion of three and four micromeres or isolation of the intact first quartet. These were classified as "spherical" or "swollen" embryos. The former were less well developed than half larvae, with solid but poorly differentiated tissues, and were either one-eyed or eyeless. Larvae with the "swollen syndrome" had almost perfectly spherical morphology, were greater than 100 μm , and showed very abnormal tissue development. The inner tissues typically were undifferentiated and a transparent space existed between the outer ectoderm and inner undifferentiated mass. These forms often had multiple eyes (Fig. 2C). The data for all experimental categories are summarized in Table I.

Deletion of one micromere (1a or 1c vs 1b or 1d)

The first two columns in Table I show the results of deletion of single first quartet micromeres at the eight-cell stage. Regardless of which micromere was deleted, 16–18% of the larvae were completely normal. Most larvae (85%) exhibited normal Muller's morphology with bilateral symmetry, so that observed abnormalities were

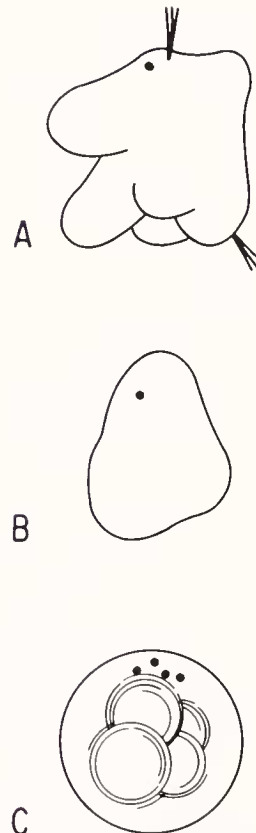


Figure 2. (A) "Three-quarter" larva. (B) "Half" larva. (C) "Swollen syndrome" larva.

Table I

Effect of deleting micromeres and macromeres on eight-cell embryos of *Hoploplana inquilina*

	-(1a or 1c) n = 61	-(1b or 1d) n = 50	-2 mics. n = 27	-3 mics. n = 29	-4 mics. n = 22	-4 mics. n = 20
Normal	10 (16%)	9 (18%)	0	0	0	0
Morphology						
Muller's	52 (85%)	37 (74%)	15 (56%)	0	0	0
3/4 Larvae	9 (15%)	11 (22%)	10 (37%)	6 (21%)	0	0
1/2 Larvae	0	2 (4%)	2 (7%)	9 (31%)	0	4 (20%)
Sphere/swollen	0	0	0	14 (48%)	22 (100%)	16 (80%)
Tufts						
Normal	38 (62%)	24 (48%)	4 (15%)	0	0	0
-Apical	15 (25%)	13 (26%)	18 (67%)	8 (28%)	1 tuft: 5 (23%)	1 tuft: 2 (10%)
-Posterior	5 (8%)	5 (10%)	0	0		
-Both	3 (5%)	8 (16%)	5 (18%)	21 (72%)	17 (77%)	18 (90%)
# Eyes						
0	1 (2%)	0	2 (7%)	8 (28%)	1 (5%)	20 (100%)
1	22 (36%)	9 (18%)	19 (70%)	19 (66%)	0	0
2	35 (57%)	37 (74%)	4 (15%)	1 (3%)	4 (18%)	0
3	3 (5%)	4 (8%)	2 (7%)	1 (3%)	8 (35%)	0
4	0	0	0	0	7 (32%)	0
5	0	0	0	0	2 (9%)	0

Minus sign indicates deletion.

in the number of eyes or tufts. Three-quarter larvae, which also exhibited fundamental bilateral symmetry, constituted 15% of embryos with 1a or 1c deleted and 22% with absence of 1b or 1d. In this category the right ventrolateral lobe was truncated or missing in two larvae, the left ventrolateral lobe in four, the dorsal lobe was deficient in one, and all lobes were abnormal in two. Only a very small number were categorized as half larvae. There was no significant difference in larval morphology related to deletion of 1a or 1c vs 1b or 1d ($\chi^2 = 1.8$, $P > 0.05$).

Deletion of one first quartet micromere resulted in loss of a tuft in approximately half of the embryos, with the apical tuft more commonly missing than the posterior tuft. A small number were missing both tufts. There was no significant difference in tuft abnormalities with respect to the particular cell deleted ($\chi^2 = 1.88$, $P > 0.05$).

Although most larvae had two eyes, there was a significantly greater proportion of one-eyed larvae when 1a or 1c was deleted (36%) versus 1b or 1d (18%) ($\chi^2 = 5.2$, $P < 0.05$). Of the single-eyed larvae, when 1a or 1c was deleted, 22 (50%) had an eye on the right, 5 (23%) were left-eyed, and 6 (27%) had a centrally located eye. Deletion of 1b or 1d resulted in 5 (56%) right-eyed and 2 (22%) left-eyed larvae. One had a central eye and in one the position of the single eye could not be determined.

Deletion of two adjacent micromeres

When two micromeres rather than one were deleted (Table 1, column 3), the number of larvae with normal

(Muller's) morphology decreased significantly, while concomitantly the number of three-quarter larvae increased ($\chi^2 = 7.09$, $P < 0.01$). Of the latter, three had truncated or missing right ventrolateral lobes, five had comparable abnormalities of the left ventrolateral lobe, one had a deficient oral hood, one had an abnormal dorsal lobe, one had all abnormal lobes, and one was missing both ventrolateral lobes. Similarly, the number of larvae with normal tufts decreased significantly ($\chi^2 = 22$, $P < 0.01$). (For the chi square analysis, the data from both kinds of single micromere deletions were pooled because the larvae from the two groups were not significantly different in morphology or tufts.)

There was a significantly greater number of one-eyed larvae with deletion of two adjacent micromeres compared with deletion of 1a or 1c ($\chi^2 = 12.02$, $P < 0.01$) and deletion of 1b or 1d ($\chi^2 = 26.3$, $P < 0.01$). The majority of these (12 or 63%) had a central eye, 3 (16%) were right-eyed, and 4 (21%) were left-eyed.

Deletion of three micromeres

A drastic effect on development was seen when three micromeres were deleted (Table 1, column 4). There were no morphologically normal larvae, and almost half fell in the highly abnormal category of spherical, swollen forms. Of the "three-quarter" larvae, one was missing the left ventrolateral lobe, two lacked both ventrolateral lobes, and in one all lobes were abnormal. Only one larva had two eyes; the remainder were almost all one-eyed or

eyeless, and 77% were missing both tufts. The number of one-eyed larvae was not significantly different between the two and three micromere experiments, but there was a significantly greater number of eyeless larvae in the latter experiments.

Isolation of the intact first quartet

Thirty-eight experiments were done in which all four macromeres were deleted leaving the four first quartet cells. Only 22 (58%) survived (Table I, column 5), and all were spherical with 95% exhibiting the "swollen syndrome." Seventy-six percent of the larvae had more than two eyes, and 77% had no tufts.

Isolation of the first quartet macromeres

The results of deleting the entire first quartet so that only the macromeres 1A–1D remain are presented in Table I, column 6. Of 105 embryos in which the 1a–1d cells were deleted, only 20 survived. These were highly aberrant; 70% were categorized as "spheres," undergoing little development beyond cleavage and no tissue differentiation. Ten percent (2) became swollen, and a small number (4) could be characterized as half larvae. None had any eyes, and 90% were tuftless.

Discussion

The results of micromere deletion experiments on *Hoploplana* corroborate the earlier four-cell deletion experiments suggesting that morphogenetic determinants are sequestered early in development, but that specific blastomeres do not receive consistently specific determinants.

Eyes and tufts

If the *Hoploplana* cell lineage conformed to the molluscan plan, only 1a and 1c should form eyes. Although deletion of one of these blastomeres results in one-eyed larvae in a statistically significant number of cases, deletion of the ventral and dorsal 1b and 1d cells also produces one-eyed larvae. Moreover, the majority have the normal two eyes. Thus, *Hoploplana* is more similar to *Bithynia* (van Dam and Verdonk, 1982) and *Lymnaea* (Morrill *et al.*, 1973), which may develop normally after deletion of first quartet micromeres, than to *Ilyanassa* (Clement, 1967), which appears to be more rigidly mosaic. The 1a and 1c blastomeres cannot be distinguished from cells 1b and 1d in the *Hoploplana* embryo; therefore the basis for the preponderance of right-eyed larvae—whether simple non-random deletion, or more complex developmental processes—cannot be determined. The occurrence of a single, centrally located eye

is probably due to the disturbed cellular topography resulting from blastomere deletion.

When two adjacent micromeres are deleted, the number of one-eyed larvae is significantly larger than when one micromere is deleted; but the proportion does not reach 100% (15% still have the normal two eyes) as would be expected if eye development involved simply the localization of eye determinants in opposite micromeres. The two eyeless larvae also suggest a more complex system than can be explained by strict cytoplasmic localization. Similarly, Morrill *et al.* (1973) found that paired eyes develop in *Lymnaea* when one, two, or three micromeres are deleted at eight cells. Because cells other than 1a or 1c can produce eyes, they concluded that eye determination involves some kind of induction and that normal development is possible only when the cleavage pattern and cell arrangements are normal.

When three micromeres are deleted in *Hoploplana*, almost all of the larvae are one eyed (66%) or eyeless (28%), and isolated first quartet macromeres never produce any eyes. Thus, at least two micromeres must be present for two eyes to form. However, isolated first quartets commonly develop supernumerary eyes, suggesting that the macromeres may play an inhibitory role in eye development. Similarly, Cather (1973) has demonstrated an inhibitory role of the polar lobe in *Ilyanassa* (a macromere derivative), in the development of cilia by first quartet cells.

As the number of micromeres deleted increases, tuft abnormalities also increase. Single micromere deletions suggest that tuft determinants are localized in the first quartet micromeres, but that tufts are equally likely to be absent upon the deletion of any blastomere. Deletion of two or more micromeres almost always results in larvae missing one or both tufts.

Symmetry

Embryos in which one or two micromeres have been deleted almost always develop bilateral symmetry, as is characteristic of Muller's and three-quarter larvae. However, following deletion of three micromeres, most of the larvae fall into the half-larva or sphere/swollen categories, exhibiting either asymmetry or radial symmetry. Thus, the determination of embryonic axes resulting in a larva with bilateral symmetry may involve an interaction between the micromeres and a central cross-furrow macromere, and a minimum number of micromeres (*i.e.*, at least 2) must contact the central macromere for axis determination to occur. In these characteristics, *Hoploplana* appears to be similar to *Patella* (van den Biggelaar and Guerrier, 1979) and *Lymnaea* (Martindale *et al.*, 1985).

Survival

Though embryos consisting of only four micromeres are much smaller than those of four macromeres, they have much greater developmental potential than the latter. Their survival rate to day five is much higher, and they differentiate structures such as eyes, cilia, and sometimes tufts, though they almost always remain spherical. Wilson (1904), Horstadius (1937), and Costello (1945) observed similar development of isolated first quartets of *Patella*, *Cerebratulus*, and *Nereis*, respectively. The swollen syndrome that characterizes this type of embryo in *Hoploplana* may result from micromeres attempting to spread over macromeres that are not there.

Isolated, intact first quartet macromeres (1A-1D embryos), on the other hand, seldom survive, and those that do are radially symmetrical and exhibit no recognizable differentiations, such as gut, eyes, or tufts. Development of eight-cell vegetal halves in *Patella*, *Cerebratulus*, and *Nereis* is similar, though these embryos sometimes gastrulated. These results are in contrast to those of first quartet deletions in *Bithynia* (van Dam and Verdonk, 1982), in which the resulting larvae were bilaterally symmetrical and differentiated many larval organs, though they were missing the head.

Conclusions

The *Hoploplana* embryo apparently develops axes of bilateral symmetry only when at least two micromeres are present, and for completely normal development to occur, at least three micromeres are required. While the blastomeres apparently do express cytoplasmic localization during early cleavage, positional differences between blastomeres also appear to play a role in divergence of developmental pathways. Therefore the polyclads, with slightly unequal cleavage, and a rather loose early embryonic determination involving cytoplasmic localization, also demonstrate some complex cellular interactions during development. These studies suggest that polyclad flatworms could be an appropriate model for an ancestral form that might have given rise to the two different developmental pathways characterizing present day higher Spiralia.

Acknowledgments

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